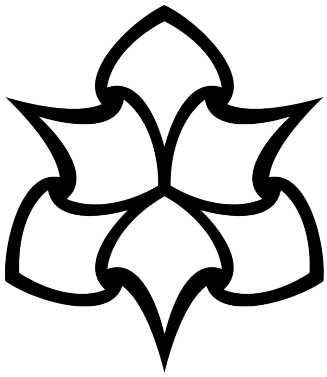

Defence responses of native and invasive plants to the native generalist vine parasite *Cassytha pubescens* – Anatomical and functional studies

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RUNNING TITLE: Native and invasive plants' responses to a vine parasite

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Summary

We investigated the responses of two invasive and two native host species to the parasitic vine *Cassytha pubescens* using glasshouse experiments. We assessed growth of the parasite and its hosts, and anatomy and functionality of haustoria. Target hosts were infected using *C. pubescens* already established on a donor host. This enabled measurement of growth in target hosts that were detached (parasite connection severed) or not from the donor host. Haustorial connections to hosts were investigated using histological methods. We tested the functionality of haustoria in one invasive and one native host using radiolabelled phosphorus (^{32}P).

After it was severed from the donor host, *C. pubescens* grew poorly on the native host, *Acacia myrtifolia.* This was likely due to a lack of effective functional haustorial development: while haustoria were firmly attached and morphologically alike those formed on the other hosts, their anatomy was different: their connections with the vascular system were not developed and there was no transfer of ^{32}P from *A. myrtifolia* to the parasite. In contrast, the other three host species supported the growth of the parasite and had fully developed haustoria. Effective transfer of ^{32}P from the invasive host to the parasite confirmed this. Our results suggest a range of defence mechanisms in *C. pubescens* hosts and are consistent with reports of strong detrimental effects on invasive hosts. Further, they amount to evidence for the potential use of a native parasite as biological control for invasive species.

Keywords: parasitic plants, ^{32}P tracer, histology, biological control, *Acacia myrtifolia*, *Leptospermum myrsinoides, Cytisus scoparius*, *Cassytha pubescens*

Introduction

Parasitic plants are significant components of natural vegetation worldwide. They affect biodiversity and ecosystem processes and services through their negative effects on native and invasive species. However, the differential responses between native and invasive host species may contribute to changes in plant community structure, and may be particularly useful to control invasive host species if they are differentially impacted (Yu *et al.* 2009; Yu *et al.* 2011; Těšitel *et al.* 2020).

While host range in parasitic plants is well documented, variation in host responses to generalist parasites has only been well studied for a few species, but has been shown for both stem and root parasites (Cameron *et al.* 2006). Differential infection rates seem to be a function of either active host selection by the parasite (Hart 1990; Kelly 1992; Callaway and Pennings 1998), or differences in the resistance/tolerance of hosts (Cameron *et al.* 2009). Despite a large host range, generalist parasites tend to preferentially utilise a subset of the species available. In the field this is most commonly observed as the disproportionate use of host species relative to species abundance (Kelly *et al.* 1988; cf. Koch *et al.* 2004) and is considered to indicate host preference by the parasite.

Resistance to parasitic plants includes several different mechanisms that generally act to prevent establishment of a functional haustorial connection between host and parasite. The extent to which haustorial development and functionality are impaired varies. Host defence responses range from full resistance (where penetration is prevented or impeded), to a continuum (high to nil) of tolerance responses (hosts traits that reduce the effect of the parasite on host fitness) (Koskela *et al*. 2002; Gurney *et al.* 2003). For example, full xylem-xylem continuity with the host is achieved by *Striga hermonthica* attached to the tolerant host *Tripsacum dactyloides*,

while some cereal cultivars can prevent effective haustorial development of the parasite (Gurney *et al.* 2003). Similarly, *Rhinanthus minor* haustoria are prevented from penetrating host xylem in *Plantago lanceolata* and *Leucnathemum vulgare* because of extra lignification or hypersensitive responses in the hosts (Cameron *et al.* 2006; Cameron and Seel 2007). Use of isotope tracing showed that *R. minor* had only very limited access to nutrients from these hosts, confirming the lack of full functionality of the haustoria (Cameron and Seel 2007).

The Australian parasitic vine *Cassytha pubescens* R.Br. is a generalist that grows on a wide range of species, usually spreading and attaching to a large number of individuals of different species. Field surveys in areas with native and invasive species, demonstrated that infection by *C. pubescens* was somewhat disproportionate to species availability, indicating slight or no host preference by the parasite (Prider *et al.* 2009; Supplementary Material Table S1; Figure S1). Pot experiments showed that when placed between a known host, an artificial plant and an empty space *C. pubescens* did not grow preferentially in any direction (Noriko Wynn unpublished data). This suggests that unlike other parasitic vine species (e.g. *Cuscuta* spp, Kelly 1992; Runyon *et al.* 2006), *C. pubescens* does not appear to detect the presence of nearby hosts.

We investigated the associations between *C. pubescens*, two invasive hosts (*Cytisus scoparius* (L.) Link and *Ulex europaeus* L.) and two native hosts (*Acacia myrtifolia* (Sm.) Wild. and *Leptospermum myrsinoides* Schltdl.). We examined growth of both the parasite (host use) and its hosts (host responses), and the anatomy of haustoria on each host. Further, we tested the functionality of the haustorial connections in one invasive (*C. scoparius*) and one native species (*A. myrtifolia*) using radiolabelled soil phosphorus (^{32}P).

Materials and Methods

Plant species

Cassytha pubescens (Lauraceae) is a perennial, rootless, stem-twining, hemi-parasitic vine native to southern Australia. Its leaves are reduced to scales, but the stem contains chlorophyll and is capable of photosynthesis (Abubacker *et al.* 2005; Prider *et al.* 2009). *Cassytha pubescens* is an obligate parasite, and has to attach to a host within 6 weeks of germination to survive (McLuckie 1924). It has a wide host range including many native Australian woody perennials and also non-native invasive perennial shrubs (Prider *et al.* 2009; Supplementary Material Table S1). Although morphologically similar to the well-studied parasitic vine *Cuscuta* spp. (Convolvulaceae), the life strategy is quite different. Whereas *Cuscuta* is a genus of annual holoparasites, in which the stem contains little or no chlorophyll (Kuijt 1969; Allen and Allen 1990), *C. pubescens* is a perennial hemiparasite that spreads mostly through vegetative growth, growing across branches within a host and spreading from one plant to another, often connected to several individuals of different species.

The woody perennial hosts tested in different experiments were two invasive shrubs, *Cytisus scoparius* (Fabaceae) and *Ulex europaeus* (Fabaceae), and two native shrubs *Acacia myrtifolia* (Fabaceae) and *Leptospermum myrsinoides* (Myrtaceae). *Cytisus scoparius* and *U. europaeus* were apparently introduced in the early 1800 as hops substitute (the former) and garden plants (Waterhouse 1988; Ireson *et al.* 2003). Both species are listed as Weeds of National Significance (Australian Weeds Committee 2012). The distribution of the four species overlaps with that of the parasite in South Australia in the open sclerophyll woodlands of the Mt Lofty Ranges around Adelaide. In these woodlands, we found *C. scoparius*, *A. myrtifolia* and *L. myrsinoides* to be amongst the species on which *C. pubescens* was most abundant and its haustoria were

firmly attached (Supplementary Material Figure S1). In field and glasshouse studies, *C. pubescens* has been shown to have strong negative effects on the growth of *U. europaeus* and *C. scoparius* but not on the native shrub *L. myrsinoides* (Prider *et al.* 2009; Cirocco *et al.* 2016, 2017, 2018). Presently there is no information about the ecophysiological responses of *A. myrtifolia* to the parasite. Field observations (summarised in Supplementary Material) report haustoria (morphologically alike those formed on other species) firmly attached, and large amounts of the parasite growing on it. However, the surveys did not determine if the parasite was also connected to other surrounding hosts that could have been supporting its growth. A greenhouse experiment (Tsang 2010) found that shortly after the connections of *C. pubescens* with the donor host were severed, the parasite growing on *A. myrtifolia* died.

Unless otherwise stated, all plant material (seeds, collected plants etc.) used in our study came from the same area in the Mt Lofty Ranges. The native host species were sourced from a local nursery (Native Flora, SA) and the invasive species obtained from stock grown by the Terrestrial Plant Ecology Laboratory, The University of Adelaide.

Experiment 1 – Growth of parasite and hosts

Experimental set up

Twenty-four individuals each of *L. myrsinoides*, *A. myrtifolia*, *U. europaeus* and *C. scoparius* were grown in 140 mm pots filled with native potting mix and a slow release native fertiliser (Osmocote, Scotts-Sierra Horticultural Products, Marysville, OH, USA), supplied at the recommended dosage, in a greenhouse in Adelaide. Sixteen individuals of each species (target hosts) were infected using tendrils from *C.*

144 *pubescens* growing on eight *C. scoparius* plants (donor host) (Shen *et al*. 2010). Two
145 individuals from each species were placed randomly around each infected *C.*
146 *scoparius* donor plant and *C. pubescens* tendrils were trained onto the new host. Eight
147 uninfected individuals of each target host species acted as controls. Plants were misted
148 twice daily for ten minutes and temperatures within the greenhouse maintained at
149 approximately 23˚C. After three months, the connection between *C. pubescens*
150 growing on the donor host and one of the target hosts of each species was severed.
151 The target hosts by then had well established growth of *C. pubescens* with well
152 attached haustoria. This created three treatments: detached (parasite connected to
153 target host only), connected (parasite connected to donor and target hosts) and control
154 (uninfected target hosts). The detached treatment examined the growth of *C.*
155 *pubescens* (and corresponding host) when growing on a single host. The connected
156 treatment examined parasite growth (and corresponding host) when utilising the
157 resource from two hosts: *C. scoparius*-*A. myrtifolia*, *C. scoparius*-*C. scoparius* , *C.*
158 *scoparius*-*L. myrsinoides* and *C. scoparius*-*U. europaeus*.
159
160 *Data collection and analyses*
161 After five months the shoot biomass of all host plants and the parasite was harvested.
162 When *C. pubescens* was separated from the host plants, the total number of haustoria
163 formed and the number of haustoria with firm connection to the host stem were
164 recorded. Parasite biomass was separated into dead and living material. Host and
165 parasite tissue were dried for 96 hours at 80 ˚C then weighed. ANOVAs were applied
166 to parasite biomass (species, four levels; treatment, two levels: connected and
167 detached) and host biomass (species, four levels; treatment: three levels: connected,
168 detached and control) using JMP 7 (SAS Institute). The Tukey-Kramer HSD test was

used to compare means where the effects of treatments were significant.

Experiment 2 - Haustoria formation – histology

The anatomy of haustoria of *C. pubescens* growing on the four different host species was studied using light microscopy. Haustoria from stems with a minimum infection time of ten weeks and a maximum stem diameter of 3 mm were harvested from three healthy individuals of *U. europaeus*, *C. scoparius*, *A. myrtifolia* and *L. myrsinoides* grown as described in experiment 1. Specimens were preserved in 2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), at 4 ˚C for four weeks to allow the fixative to penetrate the plant tissue. Specimens were then washed in 100% ethanol and dehydrated in a graduated ethanol series for 40 minutes in each 70%, 90% and 100% ethanol under vacuum. The haustoria were left under vacuum for 12 hours in a 1:1 solution of 100% ethanol and LR-White resin. Samples were embedded in 100% LR-White resin after being placed in resin for 84 hours under vacuum with resin changes every 12 hours and then set in gelatine capsules for 48 hours at 80 ˚C. Three haustoria from each species were cut into sections transverse to the stem of the host, 2 to 4 µm thick (Leica Ultracut E Ultramicrotome). Sections were floated onto slides, placed on an 80 ˚C hotplate and stained on the hotplate using 1 % Toluidine blue O in boric acid. Sections were examined under a light microscope (Olympus BX51) fitted with a camera (Colorview III Camera).

Experiment 3 - Functionality of haustoria – Transfer of radiolabelled P

To test functionality of firmly attached haustoria of *A. myrtifolia* and *C. scoparius* we compared transfer of ^{32}P between pairs of hosts connected by *C. pubescens* (Fig 1).

Experimental set up

Ten seedlings of *C. scoparius* were collected from a field site near Adelaide (35° 0'58.08"S, 138°45'58.45"E), South Australia. The seedlings were placed in 1.5 L pots with sandy loam soil, in a greenhouse for two months until established. Ten seedlings of *A. myrtifolia* were grown in 1.5 L pots in a greenhouse for six months. All plants were watered as required. The *C. scoparius* plants were infected with *C. pubescens* by placing them next to an already infected *C. scoparius* and directing the tendrils of the parasite to the stem of the target seedlings (as described above; Shen *et al.* 2010). After approximately three months, the connections between the donor host and the target seedlings were severed and the 10 newly infected *C. scoparius* plants used to similarly infect one plant each of *A. myrtifolia*. The pots containing *A. myrtifolia* plants were left for 10 weeks next to the infected *C. scoparius* plants to allow the haustoria of *C. pubescens* to develop. All plants were watered with 250 mL of reverse osmotic (RO) water three times a week and received 290 mL of full strength Hoagland's solution in the 4th week. To increase the phosphorous requirements in the hosts, in the 8th week all pots received the same amount of Hoagland's solution but with only one fifth the amount of phosphate. In the 11th week, the 10 pairs of hosts, all having several haustoria of the parasite firmly attached to both plants, were randomly assigned to two treatments (five pairs per treatment): 1) radioactive phosphate (^{32}P) injected into the soil of pots containing the *C. scoparius* host or 2) ^{32}P injected into the soil of pots with the *A. myrtifolia* host (Fig. 1). Each injected pot received 6 MBq of radioactive phosphate (carrier-free $H_3{}^{32}PO_4$) dissolved in 125 mL of RO water, divided into 5 aliquots of 25 mL each. Each aliquot was injected using a syringe with a 10 cm needle into 5 different locations in each pot to maximize the chance of it being absorbed by the host. Two weeks after injection, each pair of plants and their

parasite were harvested and divided into the following components: 1) host shoot from the pot injected with ^{32}P, 2) *C. pubescens* growing on the radio-labelled host, 3) *C. pubescens* spanning between the two hosts, 4) *C. pubescens* on the non-labelled host, 5) infected shoot of the non-labelled host, and 6) uninfected shoot of the non-labelled host (Fig. 1). Plant material was dried for 2 days at 70 ˚C and then ground to a fine powder. For each replicate, 5 mL of nitric acid was added to 0.5 g of ground plant material in a test tube, and digested overnight in a heat block at 140 ˚C (Hanson 1950). *Acacia myrtifolia* digests were centrifuged at 2000 rpm for 10 minutes to remove a milky gelatinous residue. Radioactivity was determined using 2 mL aliquots of the digests in a liquid scintillation counter (Wallac 1215 RackBeta II) by measuring the Cerenkov radiation produced by beta particles without any scintillation fluor cocktail and corrected for decay (L'Annunziata 1997).

Data analysis

One-way ANOVAs were performed using Graphpad Prism 5 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results

Experiment 1 – Growth of parasite and hosts

The amount of live biomass of *C. pubescens* was influenced by both treatment and species ($ANOVA_{interaction}$: $F_{3, 32} = 2.93$, $P = 0.049$). Live parasite biomass was significantly lower growing on a single *A. myrtifolia* individual than when growing on *C. scoparius* and *A. myrtifolia* simultaneously (Fig. 2). The growth of the parasite in the detached treatment was greatest on *C. scoparius*, and significantly higher than on either *A. myrtifolia* or *U. europaeus* but not *L. myrsinoides* (Fig. 2). Live *C. pubescens*

biomass supported by two hosts was greatest on *A. myrtifolia*, followed by *C. scoparius*, *L. myrsinoides* and *U. europaeus*. Only the live biomass on *U. europaeus* was significantly different from *A. myrtifolia* (Fig. 2). Treatment did not influence the amount of dead parasite biomass (ANOVA: $F_{1, 32} = 1.07$, $P = 0.31$), however *C. pubescens* growing on *A. myrtifolia* had more dead tissue than any of the other species ($ANOVA_{species}$: $F_{3, 32} = 14.16$, $P \leq 0.0001$; Fig. 2).

Host biomass differed between species (ANOVA: $F_{3, 48} = 128.0$, $P \leq 0.0001$). *A. myrtifolia* had the highest biomass followed by *C. scoparius*, *L. myrsinoides* and *U. europaeus* (Fig. 3). Plants in the connected treatment had lower biomass than plants in either the detached or control treatments (ANOVA: $F_{2, 48} = 7.48$, $P = 0.002$).

No differences were observed between treatments or species for either total number of haustoria on each host ($ANOVA_{species}$: $F_{3, 72} = 1.61$, $P = 0.194$; ($ANOVA_{treatment}$: $F_{1, 72} = 1.93$, $P = 0.17$), or the proportion of haustoria attached to the host stems ($ANOVA_{species}$: $F_{3, 72} = 1.61$, $P = 0.3448$; $ANOVA_{treatment}$: $F_{1, 72} = 1.93$, $P = 0.45$). *Cassytha pubescens* biomass was correlated with the proportion of haustoria that were considered to be well attached and therefore viable ($R^2 = 0.22$, Pearson two tailed test, $P = 0.001$; Fig. 4).

Experiment 2 – Haustoria formation – histology

Representative sections from the sectioned haustoria from each species are presented. All sections from the three plants per species showed the same anatomical characteristics. The haustoria formed on the two invasive species, *U. europaeus*, and *C. scoparius* had endophytes capable of penetrating host tissue. Parasite tissues are clearly observed entering the host and growing in close contact with host vascular structures (Fig. 5). Endophyte of *C. pubescens* growing on *C. scoparius* widens after

269 penetrating the host forming an oval like structure within host tissue (Fig. 5*b*, E). A
270 large proportion of the endophyte tissue is in close contact with the host xylem. The
271 early stages of a vascular core are evident, running through the middle of endophyte
272 into the haustorial tissue (Fig. 5*a*, IV). It appears that growth of the endophyte
273 structure has spread increasing the surface area in contact with host vasculature (Fig.
274 5*b*, I).

275 The anatomy of endophytes formed on *U. europaeus* was different for each of
276 the haustoria sectioned. Yet all were able to penetrate host tissues and contact host
277 vascular structures (Fig. 5*c*, *d*, I). As with the haustoria formed on *C. scoparius*, there
278 was evidence of the formation of a vascular core in dense differentiating parenchyma
279 cells running through the central body of the endophyte (Fig. 5*c*, IV). The cells of the
280 endophyte were darkly stained and appeared to form dense tissue (Fig. 5*d*, DT).

281 When grown on native host species, *C. pubescens* was able to form apparently
282 functional haustoria on *L. myrsinoides* (Fig. 6*a*) but was prevented from entering host
283 tissues when growing on *A. myrtifolia*. In the haustoria formed on *L. myrsinoides* the
284 endophyte had clearly penetrated the host tissues and formed direct luminal contact
285 with host xylem via the differentiation of xylem (Fig. 6*b*, PX). There is also evidence
286 of a hyaline rich body of cells located in the centre of endophyte tissue.
287 In contrast, *C. pubescens* growing on *A. myrtifolia* was prevented from entering host
288 tissue at the cortex, although an endophyte is present (Fig. 6*c*, *d*). There was evidence
289 of thickening host tissue where the endophyte attempted to enter the host tissue (Fig.
290 6*c*, *d*, T). At the interface between host and parasite (Fig. 6*d*, I), there are darkly
291 stained tissues; these clearly delineate the barrier between host and parasite tissues.
292 There is no evidence of a vascular core or differentiated xylem in the body of the
293 haustoria.

Experiment 3 - Functionality of haustoria – Transfer of radiolabelled P

There were significant differences in the radioactivity of plant components between the two treatments. When ^{32}P was injected into pots containing *C. scoparius*, the same level of radioactivity was detected in both *C. scoparius* and in *C. pubescens*, but only trace amounts were detected in the paired *A. myrtifolia* (ANOVA: $F_{1,2} = 12.17$, $P = 0.001$; Fig. 7*a*). This contrasted with the distribution of ^{32}P when it was injected into pots containing *A. myrtifolia*. In this case, radioactivity was detected in *A. myrtifolia* but only traces were detected in *C. pubescens* and *C. scoparius* (ANOVA: $F_{1,2} = 10.07$, $P = 0.003$; Fig. 7*b*).

Discussion

Regardless of the presence of attached haustoria and the growth of the parasite on *A. myrtifolia*, this native host resisted penetration by the parasite. In contrast, haustoria on the invasive species and on the other native species (*L. myrsinoides*) were able to penetrate host tissues successfully and, in *C. scoparius*, supported transfer of ^{32}P between host and parasite. Importantly, the relative lack of severe or lethal negative effects on *L. myrsinoides* (compared with invasive species) (Prider *et al.* 2009; Cirocco *et al*. 2015) occurs in spite of the fully developed anatomical connections we documented. This suggests that there is a range of defence mechanisms amongst hosts of *C. pubescens*.

Growth of C. pubescens *on* A. myrtifolia

Field studies have reported that *C. pubescens* is able to successfully grow on *A. myrtifolia,* and even that this is one of the species on which the parasite is more abundant (Supplementary Material Table S1). In our experiments as in field

observations we found that *C. pubescens* haustoria were as firmly attached to *A. myrtifolia* as to the other hosts. However, *C. pubescens* did not grow in high densities on *A. myrtifolia* unless it was also still attached to the donor host. Further, there was large accumulation of dead biomass on the detached plants. These results, indicate that the parasite was unable to effectively use *A. myrtifolia* as a host.

The anatomical studies showed that *A. myrtifolia* exhibited resistance by preventing the penetration of the parasitic endophyte. The localisation of the defence response indicates resistance is induced by contact and attempted penetration of host tissues by the parasite. During haustorial formation *C. pubescens* excretes a fluid which helps the parasite invade host tissues by the formation of an adhesive disk (Heide-Jørgensen 1991). This attachment mechanism is also observed in the formation of prehaustoria by *Cuscuta* spp. (Kaiser et al. 2015). Contact with this fluid may trigger the thickening of the cortical tissue in *A. myrtifolia* stems at the site of attempted parasite penetration. The parasitic vine *Cuscuta pentagona* was similarly prevented from penetrating the cortex of tomato varieties (Goldwasser *et al.* 2017). Resistance in tomato has been since attributed to hormonal signalling triggered by the parasite (Runyon *et al*. 2010). Studies of the root parasite, *Orobanche* spp., which is also prevented from penetrating tissues of resistant hosts beyond the cortex, show that the production of toxic phenols (Serghini *et al.* 2001), reinforcement of host cell walls, deposition of callose and suberisation (Perez-de-Luque *et al.* 2005; Echevarría-Zomeño *et al.* 2006) contribute to host resistance.

The lack of well-developed haustorial structure that we observed when *C. pubescens* was grown on *A. myrtifolia*, probably explains the inability of the parasite to acquire ^{32}P from this host. This confirms that *A. myrtifolia* prevents the development of functional connections by the parasite. Our results are similar to those

344 reported for the root hemiparasite *R. minor*, which absorbed different amounts of ^{15}N
345 when grown on hosts with different degrees of defence responses (Cameron and Seel
346 2007). Similar to our results, host resistance mechanisms prevented the parasite from
347 establishing functional connections with host vascular tissues. Further, the
348 concentration of ^{15}N taken up from tolerant hosts was positively correlated with
349 parasite biomass, providing additional evidence of the importance of functional
350 haustorial connections for parasite growth (Cameron and Seel 2007).
351 Biomass of *C. pubescens* was higher when growing on *A. myrtifolia* still
352 connected with the donor host, than on the detached plants. Given the lack of
353 functional haustoria when growing on *A. myrtifolia*, the parasite must have been
354 mostly relying on resources from the donor host, *C. scoparius.* This characteristic
355 complicates the study of host use by *C. pubescens*, because potentially masks native
356 host resistance or tolerance as it gives *C. pubescens* the appearance of an ability to
357 form functional haustoria and grow on resistant species such as *A. myrtifolia*. As a
358 result resistance or tolerance to *C. pubescens* may be more widespread than the host
359 range of the parasite suggests. Some native species, like *A. myrtifolia*, which could be
360 considered 'pseudo-hosts', may only provide physical support for the parasite, while
361 it moves between gaps of suitable hosts (Marquardt and Pennings 2011). While *C.*
362 *pubescens* possibly obtains little or no nutrients from these 'pseudo-hosts', they may
363 provide physical support to photosynthetic stems and facilitate its dispersal by
364 vegetative means to suitable hosts.
365
366 *Growth of* C. pubescens *on* C. scoparius*,* U. europaeus *and* L. myrsinoides
367 Comparable amounts of dead and live parasite tissue in the detached and connected
368 treatments on *C. scoparius*, *U. europaeus* and *L. myrsinoides*, demonstrates similar

parasite performance on these species. This corresponds with the anatomical similarities we observed in the development of the haustoria on these hosts. Further, the transfer of ^{32}P through the haustoria from the host *C. scoparius* to *C. pubescens* confirmed the physiological functionality of these haustoria. Generally, there is a strong association between biomass of the parasite and the transfer of resources and/or number of haustoria attached (Kelly 1992; Cameron and Seel 2007) as we observed in our first experiment (but see discussion about *A. myrtifolia* above).

Cassytha pubescens formed fully developed haustoria on the infected native *L. myrsinoides*, which also had lower biomass when infected by the parasite. Previous studies have also reported lower biomass and even some negative physiological effects on *L. myrsinoides* but detrimental effects of *C. pubescens* have been always of lower magnitude than on invasive hosts in glasshouse and field conditions (Cirocco *et al.* 2016, Prider *et al.* 2009). These effects could be attributed to incomplete haustorial connections (Cameron and Seel 2007) and/or adaptive tolerance mechanisms (Mutikainen *et al* 2000). Our results allow us to rule out the first alternative. Cirocco *et al.* (2015) proposed that the ability of *L. myrsinoides* to maintain photoprotective capacity/engagement when infected by *C. pubescens,* thereby preventing photodamage, could explain this host's tolerance. Its adaptations to low availability of water and nutrients, characteristic of plants in the sclerophyll woodlands of South Australia which contrast with the higher resource requirements of invasive species, may also contribute to its higher tolerance to reduction in resources produced by the parasite (Li *et al.* 2012). Another native host, *Acacia paradoxa*, also shows tolerance to *C. pubescens*; it supports parasite growth but host photosynthesis is not affected (Cirocco *et al.* 2017). Other native species have been observed to support the parasite (Prider *et al*. 2009; Supplementary Material Table S1, Figure S1). On the other hand,

394 our results on *A. myrtifolia* open the possibility that some of those species may
395 partially or completely prevent formation of functional of haustoria by the parasite,
396 and thus also be 'pseudo hosts'. Further research is required to determine the
397 functionality of haustoria, and parasite performance on these species, along with host
398 physiological responses to infection. This would inform our understanding of
399 ecological responses of the parasite and its many hosts (or pseudo hosts).
400
401 *Overall implications*
402 Our results suggest that the parasite does not selectively utilise invasive species over
403 natives. This generalist strategy allows the parasite to become established on host
404 species with which it has not coevolved (Koch *et al.* 2004). Importantly, however,
405 differences in resistance or tolerance of the native and invasive hosts to the parasite
406 could then induce changes in plant community structure and diversity (Yu *et al.* 2011;
407 DiGiovanni *et al.* 2017).
408 The differences in defence responses between the native and invasive hosts
409 reported here, albeit based on a small number of species, are overall consistent with
410 the prediction of the biotic resistance hypothesis (Těšitel *et al.* 2020). According to
411 this interpretation, we could speculate that the two native hosts have evolved in the
412 presence of the parasite and over time have developed suitable and different,
413 mechanisms to resist/tolerate infection (Li *et al.* 2012; Cirocco *et al*. 2016). In
414 contrast, the two invasive hosts, which were introduced to Australia less than 200
415 years ago, have not evolved defence mechanisms capable of resisting infection by the
416 novel enemy. Our results suggests a broad spectrum of responses of the native plants
417 to the native parasite. Confirming this will require a more comprehensive assessment
418 of anatomy and function of haustoria formed on native and invasive hosts, which was

beyond the scope of our study. In addition, it will be important to determine if resistance/tolerance is variable at several levels, i.e. individuals and populations, and if this variation is associated with previous coexistence, and hence coevolution, of the parasite and the host (e.g. Jerome and Ford 2002).

If differential responses between native and invasive species are proven valid for this type of vegetation, *C. pubescens* could be used as an important agent for biological control in the area (Li *et al.* 2012; Těšitel *et al.* 2020). Species used for biological control generally have high host specificity so that only the target pest is affected by the introduction of the species into a system (Myers and Bazely 2003). However, this is generally applied when introducing a further non-indigenous species into a system. The use by augmentation of a native parasite already present in the system provides a novel way to aid in control of introduced species, because infection by *C. pubescens* of invasive species has a greater effect on host health, biomass and fecundity than on the native species so far tested (Prider *et al.* 2009; Cirocco *et al.* 2016, 2018). This suggests that if used as a biological control the parasite will have little or no significant effects on native species within the system (Heer *et al.* 2018).

Further, our ^{32}P tracer technique enabled us to assess the degree of host defence responses to *C. pubescens* (similarly to the study on a root parasite of Cameron *et al.* 2006), but could also be extended for similar experiments with other stem parasites, such as the economically important Cuscuta. This technique also provides the potential to determine the relative contribution of multiple hosts simultaneously parasitised by twining stem parasites such as *C. pubescens*, by applying different tracers to the various hosts. Conversely, the impact of the parasite on its multiple hosts could also be determined.

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Conflicts of Interest

The authors declare no conflicts of interest.

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584 756.

Figure legends

Figure 1. Experimental design showing the pot containing either *Cytisus scoparius* or *Acacia myrtifolia* injected with ^{32}P (radiation symbol) and the various components harvested separately for ^{32}P analyses: (1) host shoot from the pot injected with ^{32}P, (2) *Cassytha pubescens* on the radio-labelled host, (3) *C. pubescens* spanning the two hosts, (4) *C. pubescens* on the non-labelled host, (5) infected shoot of the non-labelled host and (6) uninfected shoot of the non-labelled host.

Figure 2. Live *(a)* and dead *(b)* biomass (g) of *Cassytha pubescens* when grown on *Acacia myrtifolia* (Acacia), *Cytisus scoparius* (Cytisus), *Leptospermum myrsinoides* (Leptospermum) or *Ulex europaeus* (Ulex) and exposed to two treatments, connected to or detached from donor host. Mean + s.e. (n = 8). Different letters indicate means are significant different. Tukey-Kramer HSD, $\alpha = 0.05$.

Figure 3. Shoot biomass (g) of *Acacia myrtifolia* (Acacia), *Cytisus scoparius* (Cytisus), *Leptospermum myrsinoides* (Leptospermum) and *Ulex europaeus* (Ulex) after infection by *Cassytha pubescens* for five months in the following treatments: connected to donor host (filled bars), detached from donor host (hatched bars) and control, non-infected (clear bars). Mean + s.e. (n = 8). Different letters indicate significant differences between species. * connected treatment significantly different from detached and control. Tukey-Kramer HSD, $\alpha = 0.05$.

Figure 4. Relationship between *Cassytha pubescens* biomass and the percentage of viable haustoria over total haustoria when grown on *Acacia myrtifolia* (Acacia, circles), *Cytisus scoparius* (Cytisus, squares), *Leptospermum myrsinoides* (Leptospermum. triangles) or *Ulex europaeus* (Ulex, diamonds) and exposed to two treatments, connected (black symbols) to or detached (white symbols) from donor host.

610 Figure 5. Light microscopy of *Cassytha pubescens* haustoria on *(a) Cytisus scoparius*
611 at x 4 magnification, *(b) C. scoparius* at x 10 magnification, *(c) Ulex europaeus* at x
612 10 magnification and *(d) U. europaeus* at x 20 magnification. H, haustoria, HS, host
613 stem, PS, parasite stem, E, endophyte, HX, host xylem, PX, parasite xylem, I,
614 interface between host and parasite, IV, initial vascular core formation, DT, darkly
615 stained tissue, CL, collapsed layer, HB, hyaline body. Slides stained with 1 %
616 Toluidine blue O solution. Scale bars equal 1000 µm at x 4 magnification, 500 µm at
617 x 10 magnification and 200 µm at x 20 magnification.
618 Figure 6. Light microscopy of *Cassytha pubescens* haustoria on *(a) Leptospermum*
619 *myrsinoides* at x 10 magnification, *(b) L. myrsinoides* at x 20 magnification, *(c)*
620 *Acacia myrtifolia* at x 4 magnification and *(d) A. myrtifolia* at x 10 magnification. H,
621 haustoria, HS, host stem, PS, parasite stem, E, endophyte, HX, host xylem, PX,
622 parasite xylem, T, thickening of tissue, I, interface between host and parasite, IV,
623 initial vascular core formation, DT, darkly stained tissue, CL, collapsed layer, HB,
624 hyaline body. Slides stained with 1 % Toluidine blue O solution. Scale bars equal
625 1000 µm at x 4 magnification, 500 µm at x 10 magnification and 200 µm at x 20
626 magnification.
627 Figure 7. Radioactivity (kBq gP^{-1}) in the various plant components (see Figure 1 for
628 details of the experimental setup) when the pot containing either *Cytisus scoparius (a)*
629 or *Acacia myrtifolia (b)* was injected with ^{32}P. Means + s.d. (n=5). Different letters
630 indicate significant differences between plant components ($P \leq 0.05$). Note different
631 scales for both graphs.
632

633 Figure 1

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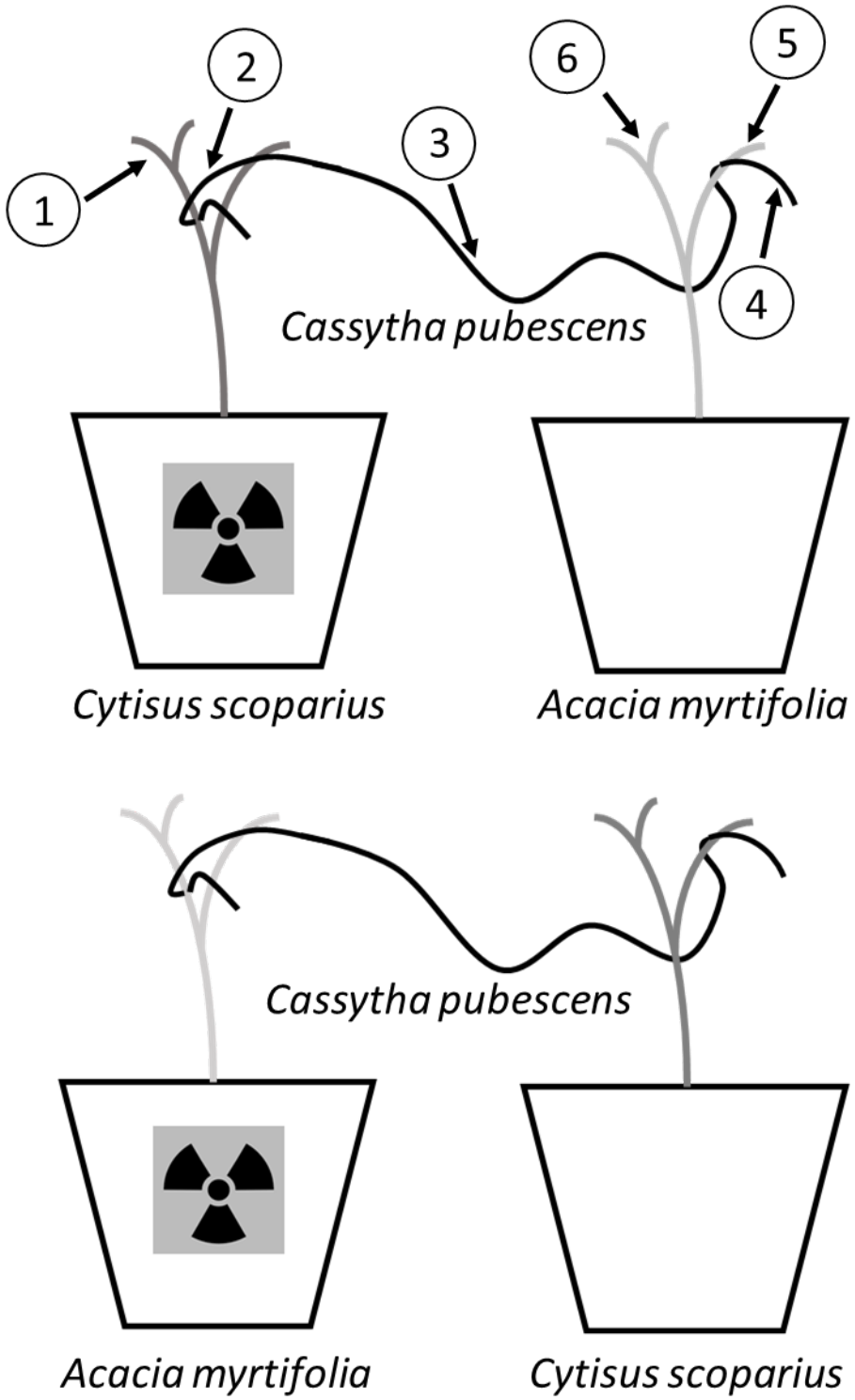

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636 Figure 2

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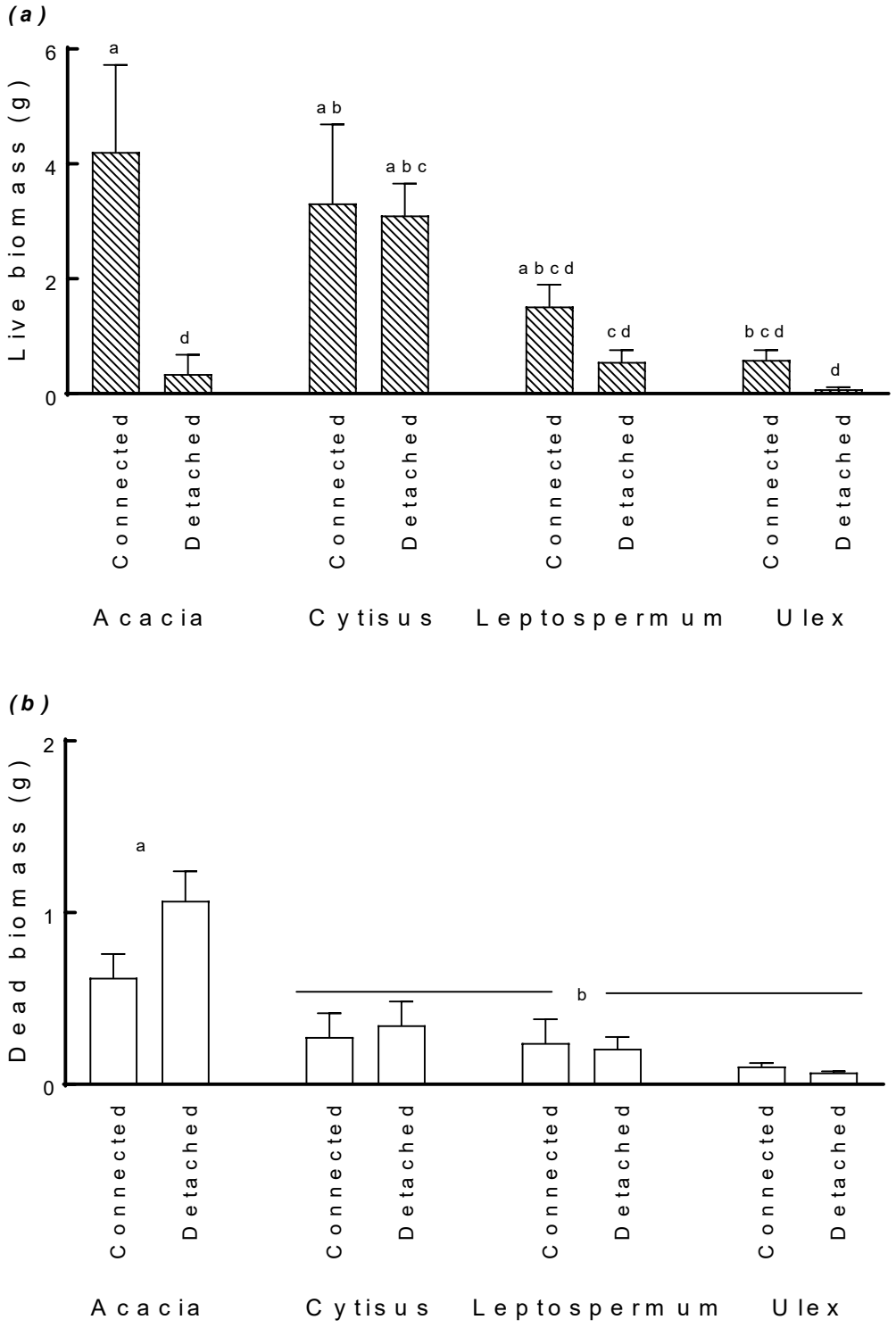

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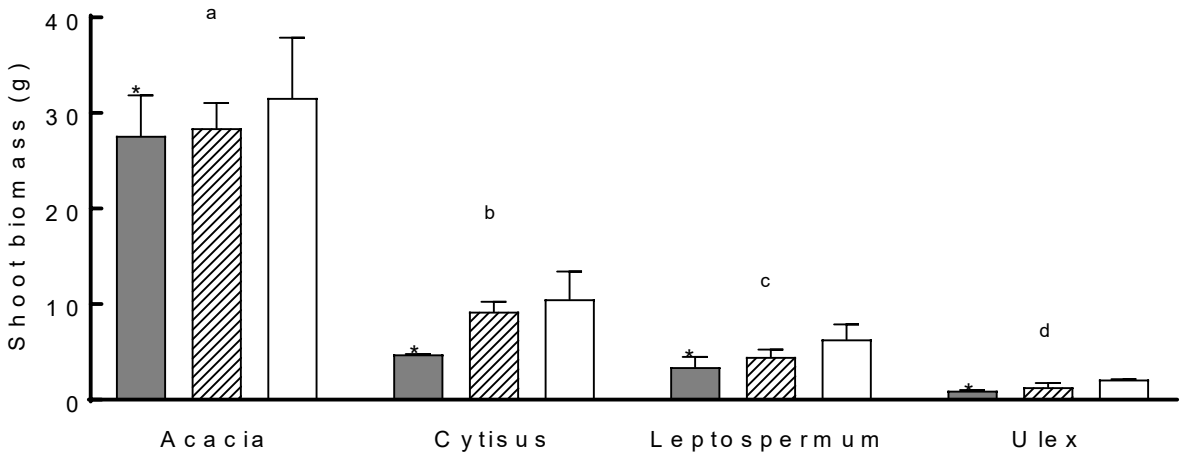

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642 Figure 4

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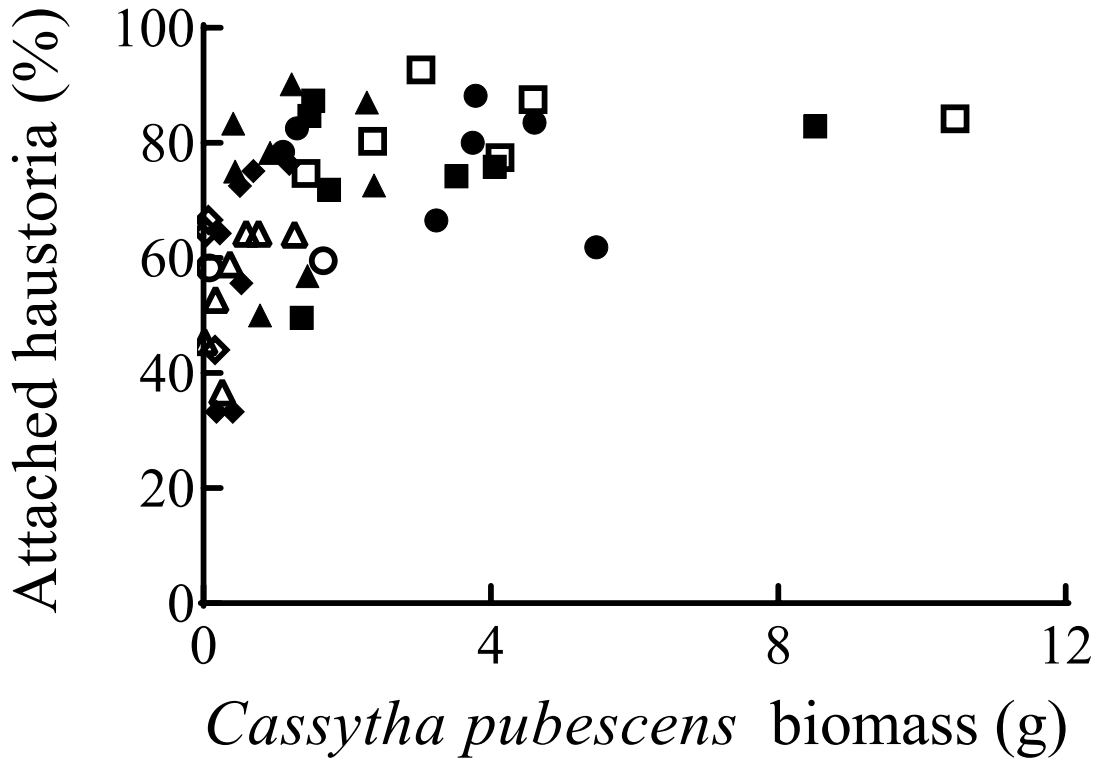

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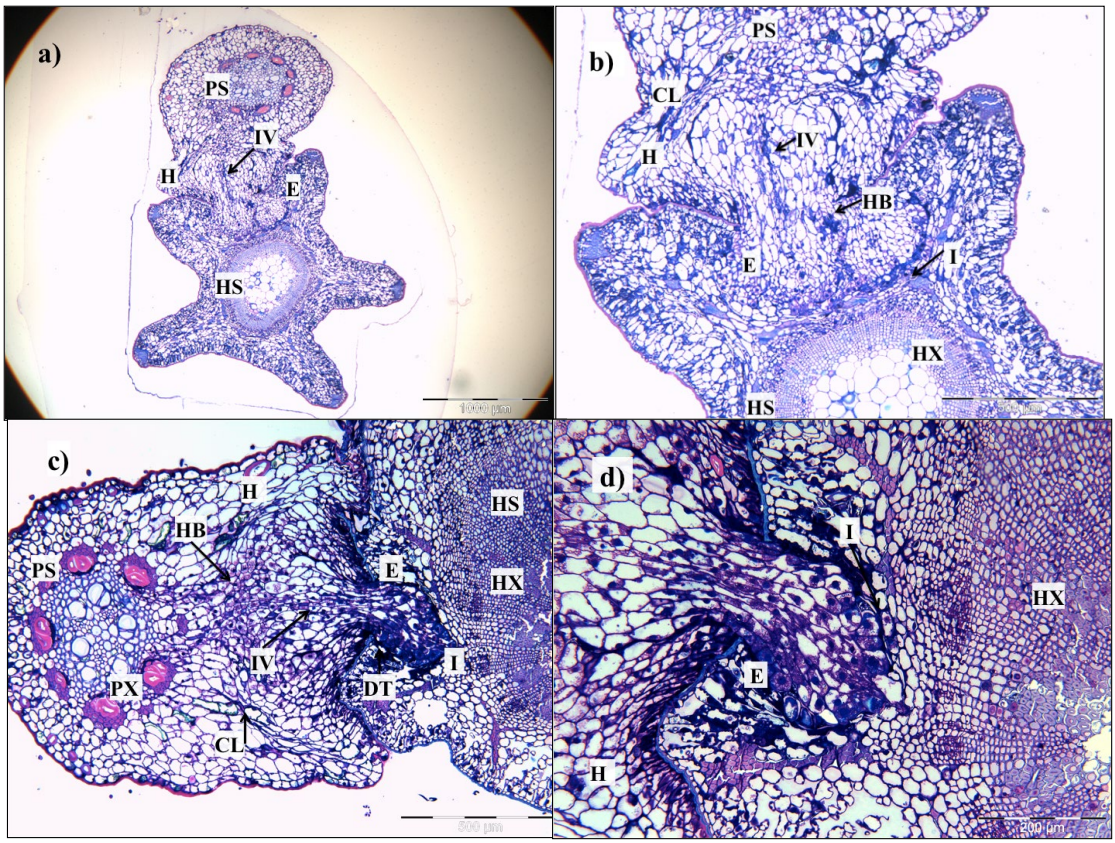

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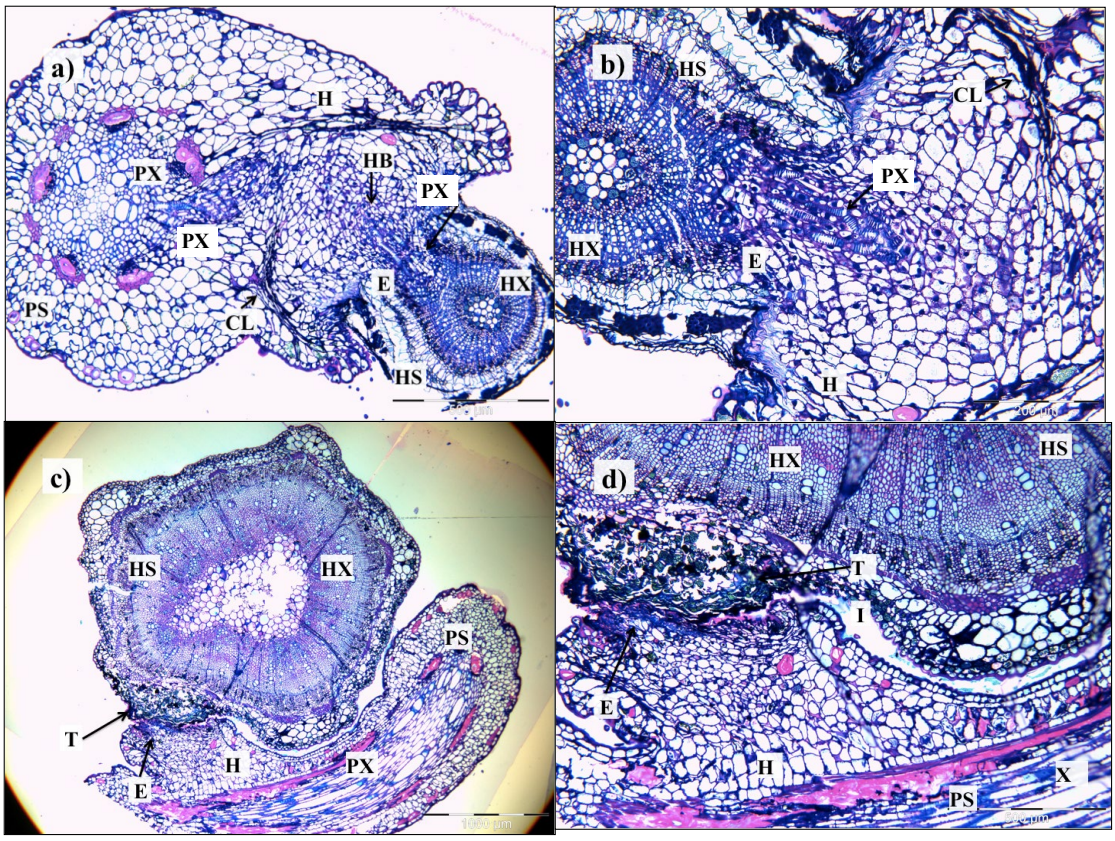
Figure 6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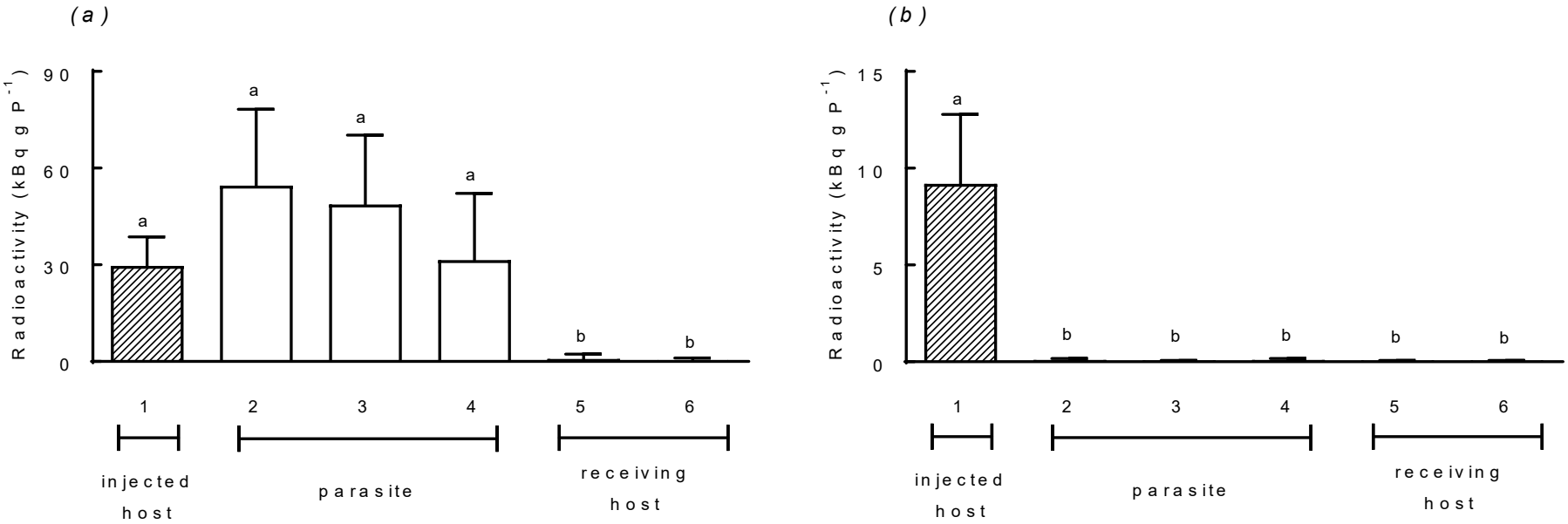
650 Figure 7



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654 Figure 7

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